AQUACULTURE

Targeted Inactivation of the Fish Aromatase Gene

Final Report

Abstract

The goal of this work is to develop gene targeting methods that can be utilized to manipulate genetic characteristics of fish and enhance aquaculture production. Although gene targeting techniques have been successfully applied to mice this technology has not been available in other species due to the absence of suitable embryonic stem (ES) cell lines. To develop this technology for use in fish, we have been working to target the inactivation of the fish aromatase genes to generate conditional sterile lines of fish in which fertility can be restored. As a first step in developing this technology, we have established zebrafish ES cell lines that remain pluripotent and germ-line competent for multiple passages in culture. ES cell cultures that remain pluripotent and maintain the capacity to contribute to the germ cell lineage of a host embryo are required in order to transfer a targeted mutation that is introduced into the cells in culture to the germ line of the fish. We have demonstrated that the zebrafish ES cells are able incorporate foreign DNA in a targeted fashion by homologous recombination and methods have been developed to identify and isolate colonies of the homologous recombinants. Work has been initiated to introduce individual colonies of the ES cells that carry a targeted mutation into host embryos to generate knockout lines of fish. Once gene targeting methods are established in zebrafish, work will be directed towards applying this technology to aquaculture species.

Introduction

The specific aims of this research are to: 1)initiate ES cell cultures derived from zebrafish embryos and maintain the cultures for multiple passages in vitro 2) construct targeting vectors that can be used to disrupt the zebrafish aromatase genes. 3) Introduce the targeting vectors into the ES cells and develop methods to isolate homologous recombinants. 4) Use the ES cell cultures to generate zebrafish aromatase knockout lines of fish.

Annual Report

During the first year of this project multiple passage germ-line competent ES cell lines were derived and characterized. During the second year, targeting plasmids were constructed, electroporation conditions for the introduction of vector DNA into the cells were optimized and methods were developed for isolating colonies of homologous recombinants. Vector DNA is introduced into the ES cells by electroporation (0.3 kV, 950 µF) using 6 x 10(6) cells suspended in PBS along with 40 µg of plasmid DNA. Using these conditions, slightly more than 50% of the embryo cells survive electroporation. The cells are plated into 4 culture dishes (100 mm), each containing a confluent monolayer of G418 resistant feeder cells. Selection is initiated by the addition of G418 (500 µg/ml) to the medium. The non-transformed cells begin to die in approximately 7 days and G418 resistant colonies are allowed to grow for 3 to 4 weeks before the homologous recombinants are selected.

To identify the ES cell colonies that have incorporated the plasmid DNA in a targeted fashion by homologous recombination, we have designed a targeting vector that contains *neo* flanked on each side by fragments of DNA that are homologous to the gene being targeted. The red fluorescent protein gene (RFP) driven by a CMV promoter is incorporated into the plasmid outside of the 5-prime homologous arm. Cells that incorporate the vector by random insertion will be RFP positive whereas the cells that have undergone homologous recombination and targeted insertion of the vector will be RFP negative. The G418 resistant

colonies are examined by fluorescence microscopy and those that are RFP negative are manually picked from the dish using a drawn-out Pasteur pipet. Initial confirmation of homologous recombination is obtained by PCR analysis of a portion of the selected colony. After the colony has been expanded, DNA is isolated from a confluent flask of cells and Southern blot analysis is performed to confirm the initial PCR results.

Final Report

Derivation of ES cell line from zebrafish gastrulas

During the course of this work an ES cell line, ZEG, was derived from zebrafish gastrula-stage embryos. The pluripotent ZEG line was established by selecting from the primary culture, individual colonies that possessed an ES-like morphology, characterized by homogeneous clusters of tightly adherent cells. Selected colonies were combined and partially dissociated to generate a suspension of small cell aggregates that were re-plated onto a fresh feeder layer of RTS34st rainbow trout spleen cells. The embryo cells were allowed to proliferate for approximately 5 days during which time the aggregates became larger while maintaining an ES-like morphology. All of the aggregates were then harvested with trypsin and re-seeded onto a fresh RTS34st monolayer. With each passage, the cell aggregates became easier to dissociate so that by passage 4, a suspension of single cells was obtained. The ZEG cells possess a fibroblast-like morphology and grow to form dense bundles of tightly packed cells on top of the feeder layer. To determine if the cells maintain the ability to contribute to the germ cell lineage of a host embryo, passage 6 ZEG cultures (6 weeks old), derived from zebrafish that possess wild-type pigmentation were injected into host embryos from the GASSI line of fish that lack melanocytes. Surviving embryos were raised to sexual maturity and crossed with non-injected GASSI mates. Two germ-line chimeras were identified from approximately 90 fish that were screened. The germ-line chimeras were identified by the production of F1 embryos that possessed body pigmentation derived from the injected cells.

Derivation of ES cell line from zebrafish blastulas

A second ES cell line (ZEB) was initiated from mid-blastula-stage embryos obtained from a transgenic line of fish that express the enhanced green fluorescent protein (EGFP) and possess wild-type pigmentation. In contrast to ZEG, all of the ZEB cell aggregates in the primary culture possessed an ES-like morphology on the RTS34st feeder cells making it unnecessary to isolate individual colonies. After 5 days, all of the ZEB cell aggregates were harvested by trypsinization and re-seeded onto a fresh feeder layer. As with the ZEG culture, the ZEB cell aggregates became easier to dissociate with each passage, eventually proliferating as a monolayer by passage 4. The ZEB cultures consisted of large epithelial-like cells that reached confluency at a low density and expressed EGFP.

To evaluate ZEB cells' ability to contribute to the germ cell lineage in vivo, cultures at passage 5 (4 weeks old) were injected into GASSI host embryos. Three days after injection, potential germ-line chimeras were identified by the presence of EGFP+ cells in the region of the gonad. Approximately 1% of the injected embryos were identified as potential germ-line chimeras in this manner. Five of the identified embryos were raised to sexual maturity and 2 were confirmed to be germ-line chimeras by the production of F1 embryos that possessed melanocyte pigmentation and expressed EGFP. This visual screening method makes it possible to rapidly examine a large number of embryos soon after injection and identify the potential chimeras, significantly reducing the number of fish that must be raised to sexual maturity for F1 screening. PCR analysis of tissues taken from adult germ-line chimeric fish revealed that the ZEB cells contribute to multiple tissues of the host embryos. In addition to the gonad, EGFP sequences were detected in muscle, liver, gut and fin indicating that ZEB behave in vivo as pluripotent ES cells. Similar results were obtained with the ZEG cell line.

We are currently working to use the ES cell lines along with the in vitro methods that we developed to isolate colonies of homologous recombinants to target mutations to the zebrafish aromatase genes and generate a

knockout line of fish.

Accomplishments or potential applications/benefits

The zebrafish ES cell lines established from this work are the first germ-line competent ES cell cultures available from any fish species. The ES cell lines along with the methods developed for targeting mutations in the cells will form the basis of a gene targeting approach that can be used to manipulate the zebrafish genome. Once established the gene targeting methods may be applied to other species of fish to improve aquaculture production, enhance disease resistance or control fertility for the purpose of biological containment.

Keywords

zebrafish, gene-targeting, ES cells, homologous recombination, aquaculture

Lay Summary

The goal of this project is to develop technology for the efficient genetic manipulation of fish in order to improve aquaculture production. To accomplish this goal, we established embryonic stem (ES) cell lines that possess the characteristics that will make them useful as tools for introducing specific alterations into the fish genome. Methods have also been developed to genetically manipulate the ES cells in the laboratory and select those cells that carry the desired alteration to be introduced into the fish embryo.

Partnerships with other institutions/individuals

Collaborations with the following scientists have been initiated:
Dr. Peter Alestrom, Norwegian School of Veterinary Medicine, Oslo, Norway.
Dr. Niels Bols, University of Waterloo, Waterloo, ON Canada
Dr. FredeRichard L. Goetz, Great Lakes Water Institute, Milwaukee, WI
Dr. J.Denry Sato, Mount Desert Island Biological Lab, Salisbury Cove, ME

Publications

Fan, L. and Collodi, P. (2004) Isolation and culture of zebrafish ES cells. In: (Lanza, et al., eds.) Handbook of Stem Cells, Academic Press (in press, Sept., 2004).
Fan, L., Crodian, J. and Collodi, P. (2004) Culture of embryonic stem cell lines from zebrafish. Methods in Cell Biology, 76, 149-158.
Fan, L., Crodian, J. and Collodi, P. (2004) Production of zebrafish germline chimeras by using cultured embryonic stem (ES) cells. Methods in Cell Biology, 77, 113-119.
Fan, L., Crodian, J., Liu, X., Alestrom, P., Alestrom, A. and Collodi, P. (2004) Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. Zebrafish 1, 21-26.
Fan, L., Alestrom, A., Alestrom, P. and Collodi, P. (2004) Development of cell cultures with competency for contributing to the zebrafish germ line. Critical Reviews in Eukaryotic Gene Expression 12, 43-51.
Fan, L., Alestrom, A., Alestrom, P. and Collodi, P. (2004) Production of zebrafish germ-line chimeras from cultured cells. In: H. Schatten, ed. Methods in Molecular Medicine 254, 289-299.
Fan, L. (2002) Progress towards cell-mediated gene transfer in zebrafish.
Briefings in Functional Genomics & Proteomics, 1, 131-138.
Collodi, P. (2002) Derivation of zebrafish ES cell cultures. Workshop on
Current Protocols in Stem Cell Biology (abstract).
Fan, L., Alestrom, A., Alestrom, P. and Collodi, P. (2002) Zebrafish embryo cell cultures for gene transfer.
Mount Desert Island Conference on Stem Cells from Land and Sea
(abstract).

Related projects resulting from this research

NIH R01 GM069384-01 Zebrafish ES cell lines for targeted mutagenesis

Patents resulting from this project
Cell culture system and methods of use, filed 12/13/02
Homologous Recombination in zebrafish cells, filed 12/10/03

Back to Research Project List (../../research_aquaculture.php)

Research Information

- **Principal Investigator**: Paul Collodi
- **Initiation Date**: March 1, 2002
- **Completion Date**: August 31, 2004
- **Affiliation**: Purdue University

Contacts

Tomas Höök (../../staff/hook.html)

Associate Director of Research
765-496-6799
thook@purdue.edu (mailto:thook@purdue.edu)

Carolyn Foley (../../staff/foley.html)
Assistant Research Coordinator
765-494-3601
cfoley@purdue.edu (mailto:cfoley@purdue.edu)

Kwamena Quagrainie (../projects/staff/quagrainie.html)
Aquaculture Specialist
765-494-4200
kquagrai@purdue.edu (mailto:kquagrai@purdue.edu)

Topics

- AQUACULTURE (/topic_aquaculture.php)
- AQUATIC INVASIVE SPECIES (/topic_ais.php)
- CLIMATE CHANGE (/topic_climate.php)
- COASTAL RESTORATION (/topic_coastal.php)
- GREAT LAKES ECOSYSTEMS (/topic_glecosystems.php)
- GREAT LAKES LITERACY (/education.php)
- MEDICINE DISPOSAL (http://web.extension.illinois.edu/unusedmeds/)
- NATURAL LAWN CARE (/l2l.php)
- NUTRIENTS (/topic_nutrients.php)

- RECREATION AND FISHERIES (/topic_recreation.php)
- RESILIENT COMMUNITIES (/topic_resilient.php)
- WATER RESOURCES (/topic_water.php)

Products

- AQUACULTURE (/products_aquaculture.php)
- AQUATIC INVASIVE SPECIES (/products_ais.php)
- CLIMATE CHANGE (/products_climate.php)
- COASTAL RESTORATION (/products_coastal.php)
- EDUCATION (/products_education.php)
- FISH CONSUMPTION (/products_fishcon.php)
- GREAT LAKES HEALTH (/products_glhealth.php)
- LAND USE PLANNING (/products_landuse.php)
- MEDICINE DISPOSAL (/products_gros.php)
- PROGRAM (/products_program.php)
- NATURAL LAWN CARE (/products_lawncare.php)
- WATER RESOURCES (/products_water.php)

Resources

- ABOUT US (/about.php)
- CHICAGO WATER WALK APP (http://www.chicagowaterwalk.org/)
- FUNDING (/funding.php)
- NEWSROOM (/newsroom)
- OTHER WEBSITES (/other_sites.php)
- PEOPLE (/staff.php)
- PHOTOS (http://iisg.photoshelter.com/)
- SOCIAL SCIENCE (/glssn.php)
- TEACHERS (/education.php)

Careers

- TEACHER TRAINING (/education.php)
- FELLOWSHIPS (/fellowships.php)
- INTERNSHIPS (/internship.php)
- SOCIAL SCIENCE (/glssn.php)

Illinois-Indiana Sea Grant
Purdue University
195 Marsteller Street

West Lafayette, IN 47907-2033
765-496-6009
iisg@purdue.edu (mailto:iisg@purdue.edu?subject=IISG Inquiry)

(http://web.extension.uiuc.edu/state/)

(http://www.seagrant.noaa.gov/)

(http://www.noaa.gov/)

(http://www.purdue.edu/)